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HUMAN GENOME EPIDEMIOLOGY (HuGE) REVIEW

CTLA-4 Gene Polymorphisms and Susceptibility to Type 1 Diabetes Mellitus: A HuGE Review and Meta-Analysis

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The authors performed a meta-analysis of 33 studies examining the association of type 1 diabetes mellitus with polymorphisms in the cytotoxic T-lymphocyte-associated antigen-4 (*CTLA-4*) gene, including the *A49G* (29 comparisons), *C*(−*318*)*T* (three comparisons), and (*AT*)*n* microsatellite (six comparisons) polymorphisms. The studies included 5,637 cases of type 1 diabetes and 6,759 controls (4,775 and 5,829, respectively, for analysis of the *A49G* polymorphism). The random-effects odds ratio for the **G* (*Ala*) allele versus the **A* (*Thr*) allele was 1.45 (95% confidence interval (CI): 1.28, 1.65), with significant between-study heterogeneity ($p < 0.001$). The effect size tended to be higher in type 1 diabetes cases with age of onset <20 years (odds ratio (OR) = 1.61), and there was a significant association between the presence of glutamic acid decarboxylase-65 autoantibodies and the **G* allele among type 1 diabetes cases (OR = 1.49). Larger studies showed more conservative results ($p = 0.011$). After exclusion of studies with fewer than 150 subjects and studies with significant deviation from Hardy-Weinberg equilibrium in the controls, the summary odds ratio was 1.40 (95% CI: 1.28, 1.54). Available data showed no strong association for the 106-base-pair allele of the microsatellite polymorphism (OR = 0.99, 95% CI: 0.64, 1.55) or the **T* allele of the *C*(−*318*)*T* polymorphism (OR = 0.92, 95% CI: 0.45, 1.89). This meta-analysis demonstrates that the *CTLA-4***G* genotype is associated with type 1 diabetes.

CTLA-4; diabetes mellitus, type 1; epidemiology; genes; meta-analysis; polymorphism, genetic

Abbreviations: bp, base pair; CI, confidence interval; CTLA-4, cytotoxic T-lymphocyte-associated antigen-4; GAD-65, glutamic acid decarboxylase-65; IDDM, insulin-dependent diabetes mellitus; OR, odds ratio.

Editor's note: *This article is also available on the website of the Human Genome Epidemiology Network (http://www.cdc.gov/genomics/hugenet/).*

GENE

The cytotoxic T-lymphocyte-associated antigen-4 (*CTLA-4*) gene is located on the long arm of chromosome 2q33. It consists of four exons and encodes a costimulatory molecule that is expressed on the surface of activated T cells (1). CTLA-4 and CD28 (also located on 2q33) are members of the immunoglobulin superfamily and bind to the B7 molecule on antigen-presenting cells. This completes the activation initiated when the antigen-specific cell-surface T-cell receptor (CD3 complex) engages the antigen bound to a major histocompatibility complex class II molecule on the surface of an antigen-presenting cell (2). CTLA-4 has a greater

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affinity for the B7 molecule than does CD28, and it down-regulates T-cell function. Therefore, it may play a crucial role in T-cell-mediated autoimmunity and thus in susceptibility to autoimmune diseases, including type 1 diabetes mellitus (3). *CTLA-4*-deficient mice rapidly develop lymphoproliferative disease with multiorgan lymphocytic infiltration and tissue destruction, with particularly severe myocarditis and pancreatitis, and die 3–4 weeks postpartum (4, 5). *CTLA-4* has also been thought to be potentially associated with a wide range of autoimmune disorders, such as autoimmune endocrinopathies, multiple sclerosis, rheumatoid arthritis, celiac disease, and primary biliary cirrhosis (6–10). It is considered the most likely candidate gene for type 1 diabetes susceptibility for the IDDM12 locus on chromosome 2q33 (11).

GENE VARIANTS

The human *CTLA-4* gene consists of four exons and three introns (11). There are at least three well-studied polymorphic markers that have drawn the most attention: a C-to-T substitution at position −318 (*C(−318)T*) of the promoter region (12), an A-to-G transition at position 49 of exon 1 (*A49G*) which causes a threonine-to-alanine substitution in codon 17 of the leader peptide (A17T) (13), and an (*AT*)*n* dinucleotide repeat polymorphism located in the 3′ untranslated region of exon 4 (14). Most epidemiologic studies have addressed the *A49G* polymorphism. There is evidence for strong linkage disequilibrium between these three polymorphisms (13, 15–17). The **T* allele of the *C(−318)T* polymorphism tends to occur with the **A* allele of the *A49G* polymorphism in European populations (15). The 106-base-pair (*106bp*) allele of the dinucleotide repeat polymorphism is linked with the **G* allele of the *A49G* polymorphism (13, 16, 17), but the *106bp* allele is rather uncommon relative to the **G* allele of the *A49G* polymorphism. The 86-base-pair (*86bp*) allele of the dinucleotide repeat polymorphism is linked with the **A* allele of the *A49G* polymorphism.

Some data on the functional significance of *CTLA-4* polymorphisms are available. The (*AT*)*n* repeat may affect RNA stability, as it has been demonstrated for the long repeats product (18). The **G* allele has been related to the strength of down-regulation of T-cell activation (19, 20) and to reduced control of T-cell proliferation (20).

Many molecular epidemiologic studies have evaluated the potential role of *A49G* (13, 21–47), *C(−318)T* (31, 34, 44, 48), and the (*AT*)*n* repeat (31, 44, 49–52) in susceptibility to type 1 diabetes. Given the amount of accumulated data, it is important to perform a quantitative synthesis of the evidence.

DISEASE

Type 1 diabetes mellitus (formerly called insulin-dependent diabetes mellitus (IDDM)) is an organ-specific autoimmune disorder characterized by the T-cell-mediated destruction of the insulin-secreting β cells of the pancreatic islets of Langerhans (53). It affects approximately 35 million people worldwide, and its incidence exhibits significant geographic and racial variation, ranging from more than 35/100,000 population/year in Finland and Sardinia (Italy) to less than 3/100,000 population/year in Asian countries, including Japan, South Korea, and China. In most other Caucasian populations in Europe and America, incidence rates are moderate (10–20/100,000/year) (54). New diagnoses peak around puberty (55). Type 1 diabetes is the second most common chronic childhood disease after asthma (56). Incidence rates are similar for males and females, although a female preponderance has been noted in low-risk populations such as the Japanese (57). There is also seasonal variation, with higher rates being observed in the winter (58). A list of Internet sites pertaining to type 1 diabetes can be found on the website of the Human Genome Epidemiology Network (http://www.cdc.gov/genomics/hugenet/).

The pathogenesis of type 1 diabetes includes a combination of multigenic predisposition and environmental factors. Viruses (59–62) such as Coxsackie B, mumps (63), and rubella (64) have been implicated as possible initiators, accelerators, or precipitators of the disease. Among postulated genetic factors, two regions have been well characterized: the major histocompatibility complex on chromosome 6p21 (IDDM1) (65, 66) and the insulin region on chromosome 11p15.5 (IDDM2) (67–70). Many other susceptibility loci have been proposed after complete or partial genome scans (IDDM3–15) (71–73), but the exact genes responsible have not yet been established. IDDM12, located on chromosome 2q33, is one of the confirmed type 1 diabetes susceptibility loci (13). This 300-kilobase region is known to contain at least three genes: *CD28*, *CTLA-4*, and the inducible costimulatory molecule (*ICOS*) gene. Genetic and physical mapping has suggested that *CTLA-4* or a gene in close proximity to it may be involved in susceptibility to type 1 diabetes (74).

MATERIALS AND METHODS

Identification and eligibility of relevant studies

We considered all studies that examined the association of the three major *CTLA-4* polymorphisms with type 1 diabetes. Sources included MEDLINE and EMBASE (search last updated in October 2004). The search strategy was based on combinations of the terms ''CTLA-4,'' ''cytotoxic T-lymphocyte-associated antigen-4,'' ''CD152,'' and ''diabetes.'' Reference lists in retrieved articles were also screened.

Nonfamilial case-control studies were eligible if the researchers had determined the distribution of genotypes for any of these polymorphisms in type 1 diabetes cases and disease-free controls. We included only published manuscripts, without any language restriction. We set no restriction on the source of controls (general population, clinic, or hospital). We excluded studies with family-based designs in which the analysis was based on linkage considerations.

Data extraction

Two investigators independently extracted data, discussed disagreements, and reached consensus on all items. The following information was sought from each report:

authors, journal and year of publication, country of origin, selection and characteristics of cases and controls, demographic data, racial descent of the study population (European, Asian, North African/Middle Eastern, Sub-Saharan African, or Pacific Asian), numbers of eligible and genotyped cases and controls, and genotype distributions in cases and controls and available subgroups thereof. Furthermore, we examined whether matching had been used; whether there was specific mention of blinding of the genotyping personnel to the clinical status of subjects; whether the genotyping method used had been validated; and whether genotype frequencies in control groups were in Hardy-Weinberg equilibrium.

Meta-analysis

For all three polymorphisms, we based the primary analysis on the contrast of alleles in order to detect overall differences. Where sufficient data were available, we also examined the contrast between the two groups of homozygotes and contrasts of each group of homozygotes with the remaining subjects for the *A49G* polymorphism.

We used the odds ratio as the metric of choice. For each genetic contrast, we estimated between-study heterogeneity across all eligible comparisons using the χ^2-based Cochran's Q statistic (75). Heterogeneity was considered significant at $p < 0.10$ (75). We also report I^2 metrics, which quantify heterogeneity irrespective of the number of studies (76). Large heterogeneity is claimed for I^2 values of $\geq$75 percent (76). Data were combined using both fixed-effects (Mantel-Haenszel) and random-effects (DerSimonian and Laird) models. Random effects incorporate an estimate of the between-study variance and provide wider confidence intervals, when the results of the constituent studies differ among themselves. Random effects are more appropriate when heterogeneity is present (75). Unless stated otherwise, random-effects estimates are reported here. In analyses of subgroups, we estimated odds ratios according to racial descent and age of onset (early onset (typically <20 years) vs. late onset (typically $\geq$20 years)). We also evaluated whether carriage of the **G* allele was associated with glutamic acid decarboxylase-65 (GAD-65) autoantibody positivity among patients with type 1 diabetes. Insufficient data were available to test for associations with other clinical or laboratory features of type 1 diabetes.

In sensitivity analyses, we excluded studies in which controls violated Hardy-Weinberg equilibrium. We performed cumulative meta-analysis and recursive cumulative meta-analysis to evaluate whether the summary odds ratio for the main analyses changed as more data accumulated (77, 78). We used inverted funnel plots and the Begg-Mazumdar publication bias diagnostics (nonparametric τ correlation coefficient) (79) to evaluate whether the magnitudes of the observed associations were related to the variance of each study.

Analyses were conducted in SPSS, version 12.0 (SPSS, Inc., Chicago, Illinois), StatXact (Cytel, Inc., Boston, Massachusetts), and Meta-Analyst (Joseph Lau, Boston, Massachusetts). All p values presented are two-tailed.

META-ANALYSIS RESULTS

Eligible studies

A total of 1,050 articles were originally screened. Thirty-three eligible studies examining the relation between *CTLA-4* polymorphisms and susceptibility to type 1 diabetes were identified (13, 21–52) (table 1). Twenty-eight studies contained data for the *A49G* polymorphism (13, 21–47), three contained data for the *C(−318)T* polymorphism (31, 44, 48), and six contained data for the *(AT)n* repeats (31, 44, 49–52). One of the eligible studies included subjects from two different racial groups (33), so a total of 29 comparisons were considered for the *A49G* polymorphism. There was considerable diversity of ethnic groups. Eligibility criteria for patients are shown in table 1. Controls were healthy subjects who were described as normoglycemic and/or nondiabetic, although varying details were presented regarding the extent of testing that had been done to exclude controls with impaired glucose tolerance and diabetes (table 1). Eleven studies (21, 26, 35–37, 39–42, 45, 47) also excluded subjects with a family history of diabetes from the control group.

One study matched for age (43); one study matched for age and gender (27); two studies matched for age, gender, and geographic region (42, 50); and one study matched for geographic region (36). No matching was reported in the other investigations. Polymerase chain reaction methods were used for genotyping in all of the studies. No researchers mentioned explicit blinding of the personnel who performed the genotyping. In five studies, the distribution of genotypes in the control group deviated significantly from Hardy-Weinberg equilibrium (27, 37, 38, 42, 45).

The *A49G* polymorphism

Meta-analysis database. The eligible studies for analysis included a total of 5,637 cases with type 1 diabetes and 6,759 controls, of which 4,775 cases and 5,829 controls were available for analysis of the *A49G* polymorphism (table 2). Genotype data were available for 4,615 cases and 5,629 controls, because Ihara et al. (31) provided only allele frequencies. The pooled frequency of the **G* allele was 43.3 percent among control subjects (by race, frequencies were 55.4 percent, 36.2 percent, 33.6 percent, 20.6 percent, and 45.2 percent among controls of Asian, European, North African/Middle Eastern, Sub-Saharan African, and Pacific Asian descent, respectively). The overall pooled prevalence of *G/G* homozygosity was 20.4 percent (33.4 percent, 12.8 percent, 8.9 percent, 5.7 percent, and 22.3 percent in the five racial descent groups, respectively). The overall pooled prevalence of *G/A* heterozygosity was 44.8 percent (44.1 percent, 46.8 percent, 49.4 percent, 31.2 percent, and 45.7 percent in the five racial descent groups, respectively).

Data synthesis. The summary odds ratio suggested a 1.45-fold increase in susceptibility to type 1 diabetes among persons with the **G* allele, a finding that was highly statistically significant ($z = 5.72$, $p < 0.001$), but there was significant between-study heterogeneity ($p < 0.001$ for heterogeneity; $I^2 = 78$ percent) (table 3; figure 1). A sensitivity

TABLE 1. Characteristics of studies included in a meta-analysis of polymorphisms in the cytotoxic T-lymphocyte-associated antigen-4 (*CTLA-4*) gene and susceptibility to type 1 diabetes mellitus

Country(ies)	Racial descent	Selection/characteristics of cases and controls		No. of eligible subjects		Authors and year of study (reference no.)
		Cases	Controls	Cases	Controls	
Belgium	European	Persons with type 1 diabetes from the Belgian Diabetes Registry	Ethnically matched subjects	483	529	Nistico et al., 1996 (13)
United States	European	Persons with type 1 diabetes; age at onset <18 years	Nondiabetic persons aged >18 years who were HLA-DR3*- or HLA-DR4-positive; absence of HLA-DR2	158	80	Owerbach et al., 1997 (52)
Germany and Canada	European	Persons with type 1 diabetes; age at onset 2–33 years (mean = 17.9 years)	Healthy persons from Germany and Canada with no family history of diabetes or autoimmune disease	293	325	Donner et al., 1997 (21)
Belgium	European	Persons with type 1 diabetes aged <40 years from the Belgian Diabetes Registry who fulfilled NDDG* criteria; mean age = 20 years	Blood donors, seemingly healthy paramedical personnel, and children with minor trauma seen in the emergency department (mean age = 20 years (SD*, 11))	525	530	Van der Auwera et al., 1997 (22)
Poland	European	Children with type 1 diabetes; mean age at onset = 9.5 years (range, 0.4–17.5 years)	Healthy blood donors from the same area	192	136	Krokowski et al., 1998 (23)
France	European	Adult-onset patients with insulin-dependent diabetes mellitus; 62.5% males; mean age at onset = 24.9 years	Healthy blood donors	112	100	Djilali-Saiah et al., 1998 (24)
Japan	Asian	Persons with type 1 diabetes per clinical features and laboratory data (islet cell antibodies and postprandial serum C-peptide); mean age at onset = 24.5 years (range, 1–69 years)	Randomly selected healthy subjects aged 18–71 years	173	425	Awata et al., 1998 (25)
Japan	Asian	Unrelated persons with type 1 diabetes who had sudden onset of severe symptoms or rapid progress to overt diabetes and were dependent on exogenous insulin; mean age at onset = 25.9 years	Unrelated persons with no clinical evidence or family history of diabetes mellitus or any autoimmune disease	110	200	Yanagawa et al., 1999 (26)
Japan	Asian	Persons with insulin-dependent type 1 diabetes according to 1997 ADA* criteria; 42.7% males; mean age at onset = 34 years (SD, 2); mean age = 44 years (SD, 2)	Healthy persons matched on age and sex	117	141	Hayashi et al., 1999 (27)†
Japan	Asian	Persons with type 1 diabetes aged 1–71 years who were diagnosed according to NDDG criteria	Randomly selected healthy subjects aged 18–71 years	111	445	Abe et al., 1999 (28)
Japan	Asian	Persons with type 1 diabetes per clinical and laboratory features (anti-GAD-65* level >1.2 U/ml, urinary C-peptide level <20 μg/day); 38.7% males; mean age at onset = 21.8 years (SD, 18.8); age ≤26.4 years	Healthy subjects; 44.9% males	74	107	Takara et al., 2000 (29)

Table continues

TABLE 1. Continued

Country(ies)	Racial descent	Selection/characteristics of cases and controls		No. of eligible subjects		Authors and year of study (reference no.)
		Cases	Controls	Cases	Controls	
China	Asian	Persons with type 1 diabetes per NDDG criteria; 50.6% males; age at onset 0.3–15.0 years (mean = 7.1 years (SD, 3.7))	Randomly selected normal adults aged >60 years with neither clinical nor laboratory evidence of diabetes and normal fasting plasma glucose levels	253	91	Lee et al., 2000 (30)
Sweden	European	Persons with type 1 diabetes aged 0–34 years	Age-, region-, and sex-matched normal subjects	616	502	Lowe et al., 2000 (50)
Japan	Asian	Persons with type 1 diabetes	Healthy controls	236	390	Ban et al., 2001 (51)
Japan	Asian	Persons with type 1 diabetes per NDDG criteria; mean age at onset = 7.9 years (SD, 5.5) (range, 0–22 years); 60.6% males	Normal children; 48.5% males	160	200	Ihara et al., 2001 (31)
Tunisia	North African	Persons with type 1 diabetes aged 1–15 years (mean age = 10.3 years)	Normal children with no autoimmune disorders	74	48	Kamoun Abid et al., 2001 (32)
China	Asian	Persons with type 1 diabetes per NDDG criteria; 70.3% males	Healthy children aged 0.3–15 years (mean age = 7.4 years (SD, 3.2)) with no islet cell antibodies; 50.5% males	350	420	Osei-Hyiaman et al., 2001 (33)
Ghana	Sub-Saharan African	Persons with type 1 diabetes recruited from pediatric units; 52.7% males	Healthy persons aged 0.3–15 years (mean age = 6.9 years (SD, 4.2)); 50.2% males	182	201	Osei-Hyiaman et al., 2001 (33)
Northern Ireland	European	Persons with type 1 diabetes with age at onset <15 years from a prospective register (diagnosis 1997–1999)	Randomly sampled healthy schoolchildren aged 12–15 years	130	307	McCormack et al., 2001 (34)
China	Asian	Persons with type 1 diabetes per NDDG criteria; 42.9% males; age at diagnosis 0.3–15.8 years (mean = 7.2 years (SD, 3.8))	Healthy siblings of patients; 46.2% males	347	260	Lee et al., 2001 (48)
Japan	Asian	Persons with type 1 diabetes per WHO* Study Group criteria; 40.0% males; age at onset 0.6–16 years	No personal or family history of autoimmune disease	125	200	Kikuoka et al., 2001 (35)
Japan	Asian	Persons with type 1 diabetes per NDDG criteria; median age at onset, 9.0 years (range, 0–64 years)	Healthy schoolchildren aged ~10 years	206	200	Bassuny et al., 2002 (49)
Italy	European	Adults with latent autoimmune diabetes; 25.5% males; mean age at diagnosis = 51 years (range, 25–68 years)	Healthy subjects with no family history of diabetes, matched geographically; 49.4% males; median age, 48 years (range, 22–62 years)	80	85	Cosentino et al., 2002 (36)
France	European	Persons with type 1 diabetes per WHO criteria; 46.3% males; mean age = 29 years (range, 5–66 years); mean disease duration = 12 years	Nondiabetic subjects with no family history of type 1 diabetes; 28.6% males; mean age = 35 years (range, 20–63 years)	134	273	Fajardy et al., 2002 (37)†
Czech Republic	European	Persons with type 1 diabetes per WHO criteria from an outpatient pediatric clinic; age at diagnosis <15 years (mean = 7.6 years (SD, 3.8)); 48.9% males	Nondiabetic children undergoing minor surgical interventions or surgery for hernias; mean age = 8.5 years (SD, 3.9)	305	289	Cinek et al., 2002 (38)†

Table continues

TABLE 1. Continued

Country(ies)	Racial descent	Selection/characteristics of cases and controls		No. of eligible subjects		Authors and year of study (reference no.)
		Cases	Controls	Cases	Controls	
China	Asian	Persons with type 1 diabetes per ADA criteria	Randomly selected blood donors with no family history of diabetes or other autoimmune disorders	31	36	Ma et al., 2002 (39)
Germany	European	Persons with type 1 diabetes attending endocrine and diabetes outpatient clinics	Healthy adults with no family record of diabetes or autoimmune disease	176	220	Wood et al., 2002 (41)
United States	Pacific Asian	Persons with type 1 diabetes per ADA criteria who were born in the Philippines	Normal subjects with no family history of diabetes	90	94	Klitz et al., 2002 (40)
France	European	Persons with type 1 diabetes per WHO criteria; mean age at diagnosis = 13.3 years (range, 5–38 years); mean disease duration = 32 years (range, 1–37 years); 54.8% males	Normal blood donors, age-, sex-, and geographically matched; no personal or family history of diabetes or other autoimmune disease	62	84	Ongagna et al., 2002 (42)†
Japan	Asian	Persons with type 1 diabetes per Japan Diabetes Society and ADA criteria; age at onset <16 years; 38.1% males	Nondiabetic age-matched subjects	97	60	Mochizuki et al., 2003 (43)
Morocco	North African	Persons with type 1 diabetes	Healthy subjects from the same geographic area	118	114	Bouqbis et al., 2003 (44)
Lebanon	Middle Eastern	Persons with type 1 diabetes with acute onset and continuous insulin dependence; nonobese; age <26 years (mean age = 14 years (SD, 5.8)); 48.9% males; mean age at onset = 8.9 years	Healthy persons aged >25 years who were anti-GAD-65-negative, with no family history of type 1 diabetes	190	96	Zalloua et al., 2004 (45)†
Japan	Asian	Persons with type 1 diabetes per ADA criteria; 47.4% males; mean age at onset = 22.0 years (range, 1–66 years)	Healthy persons with no family history of diabetes	116	114	Ide et al., 2004 (47)
Estonia	European	Persons with type 1 diabetes per the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus; 49.3% males; mean age = 39 years	Blood donors who were healthy according to medical record data	69	158	Haller et al., 2004 (46)

* HLA, human leukocyte antigen; NDDG, National Diabetes Data Group; SD, standard deviation; ADA, American Diabetes Association; anti-GAD-65, autoantibodies to glutamic acid decarboxylase-65; WHO, World Health Organization.

† Study in which the control group genotypes deviated from Hardy-Weinberg equilibrium.

analysis excluding one study that was clearly an outlier (42) yielded a summary odds ratio of 1.36 (95 percent confidence interval (CI): 1.24, 1.51; $p < 0.001$). Effect sizes were consistent across subgroups of differing racial descent (for patients of Asian descent, odds ratio (OR) = 1.41, $p < 0.001$; for patients of European descent, OR = 1.54, $p < 0.001$; data on other racial groups were sparse). There was still highly significant between-study heterogeneity within racial descent subgroups. When the five studies that deviated significantly from Hardy-Weinberg equilibrium were excluded, the random-effects odds ratio remained 1.44 (95 percent CI: 1.31, 1.59), but heterogeneity decreased ($Q = 52.84$, $I^2 = 56$ percent), even though the heterogeneity was still formally statistically significant ($p < 0.001$).

Analyses of genotypes suggested that *G/G* homozygosity more than doubled the risk of type 1 diabetes ($p < 0.001$, with substantial heterogeneity; $I^2 = 71$ percent), which is consistent with a codominant (per-allele) model. We found similar effect sizes in recessive and dominant models, with considerable heterogeneity ($I^2 = 68$ percent and $I^2 = 90$ percent, respectively). Results for all of these comparisons were consistent across racial descent subgroups (table 3).

In studies or subgroups of patients with type 1 diabetes and age of onset <20 years (15 contrasts with 11,768 alleles;

TABLE 2. Distribution of genotypes of the *A49G* polymorphism in studies of the cytotoxic T-lymphocyte-associated antigen-4 (*CTLA-4*) gene and susceptibility to type 1 diabetes mellitus

Authors and year of study (reference no.)	Racial descent	*G/G*		*G/A*		*A/A*		Frequency of the **G* allele (%)	
		No. of cases	No. of controls	No. of cases	No. of controls	No. of cases	No. of controls	Cases	Controls
Nistico et al., 1996 (13)	European	74	51	248	242	161	236	40.9	32.5
Donner et al., 1997 (21)	European	55	41	147	149	91	135	43.9	35.5
Van der Auwera et al., 1997 (22)	European	75	53	269	241	181	236	39.9	32.7
Krokowski et al., 1998 (23)	European	60	21	95	76	37	39	56.0	43.4
Djilali-Saiah et al., 1998 (24)	European	34	16	41	37	37	47	48.7	34.5
Awata et al., 1998 (25)	Asian	72	170	80	197	21	58	64.7	63.2
Yanagawa et al., 1999 (26)	Asian	45	78	46	88	19	34	61.8	61.0
Hayashi et al., 1999 (27)	Asian	54	72	42	47	21	22	64.1	67.7
Abe et al., 1999 (28)	Asian	50	177	45	207	16	61	65.3	63.0
Takara et al., 2000 (29)	Asian	33	30	25	43	16	34	61.5	48.1
Lee et al., 2000 (30)	Asian	150	37	85	45	18	9	76.1	65.4
Ihara et al., 2001 (31)	Asian	ND*	ND	ND	ND	ND	ND	70.6	57.3
Kamoun Abid et al., 2001 (32)	North African	32	11	38	28	4	10	68.9	52.1
Osei-Hyiaman et al., 2001 (33)	Asian	74	42	166	177	110	201	44.9	31.1
	Sub-Saharan African	9	11	67	61	106	129	23.4	20.6
McCormack et al., 2001 (34)	European	21	58	69	151	40	98	42.7	43.5
Kikuoka et al., 2001 (35)	Asian	57	78	62	88	6	34	70.4	61.0
Cosentino et al., 2002 (36)	European	4	5	55	40	21	40	39.4	29.4
Fajardy et al., 2002 (37)	European	17	31	76	146	41	96	41.0	38.1
Cinek et al., 2002 (38)	European	57	50	125	133	123	106	39.2	40.3
Ma et al., 2002 (39)	Asian	15	8	11	9	5	19	66.1	34.7
Klitz et al., 2002 (40)	Pacific Asian	38	21	34	43	18	30	61.1	45.2
Wood et al., 2002 (41)	European	33	26	84	95	59	99	42.6	33.4
Ongagna et al., 2002 (42)	European	49	14	10	27	3	43	87.0	32.7
Mochizuki et al., 2003 (43)	Asian	44	21	36	27	17	12	63.9	57.5
Bouqbis et al., 2003 (44)	North African	7	8	52	47	59	59	28.0	27.6
Zalloua et al., 2004 (45)	Middle Eastern	24	4	75	53	91	39	32.4	31.8
Haller et al., 2004 (46)	European	22	23	29	85	18	50	52.9	41.5
Ide et al., 2004 (47)	Asian	56	34	49	59	11	21	69.4	55.7

* ND, no data (no genotype data available).

see Web table 1, available on the websites of the *Journal* (http://www.aje.oupjournals.org) and the Human Genome Epidemiology Network (http://www.cdc.gov/genomics/hugenet/)), the random-effects odds ratio was 1.61 (95 percent CI: 1.28, 2.02; $Q = 92.99$; $I^2 = 85$ percent) (figure 2). In studies or subgroups of patients with late onset (13 contrasts

TABLE 3. Summary odds ratios from a meta-analysis of various contrasts of polymorphisms in the cytotoxic T-lymphocyte-associated antigen-4 (*CTLA-4*) gene and susceptibility to type 1 diabetes mellitus*

Contrast and racial descent group	No. of studies	Total sample size (*n*)	Random effects	95% CI†	*Q*	Fixed effects	95% CI
**G* allele vs. **A* allele	29	21,950	1.45	1.28, 1.65	128.0	1.38	1.31, 1.46
Asian	12	8,334	1.41	1.16, 1.72	42.54	1.39	1.26, 1.52
European	12	11,200	1.54	1.24, 1.91	75.12	1.40	1.29, 1.51
G/G genotype vs. A/A genotype	28	5,948	2.08	1.63, 2.67	92.44	1.97	1.74, 2.22
Asian	11	2,162	1.87	1.28, 2.72	29.09	1.84	1.49, 2.27
European	12	2,927	2.08	1.46, 2.95	42.67	1.93	1.64, 2.27
G/G genotype vs. other genotypes	28	10,604	1.68	1.39, 2.03	83.87	1.60	1.44, 1.76
Asian	11	3,796	1.52	1.20, 1.92	24.20	1.47	1.27, 1.70
European	12	5,600	1.82	1.30, 2.55	49.95	1.69	1.45, 1.95
Other genotypes vs. *A/A* genotype	28	10,604	1.76	1.30, 2.39	269.1	1.83	1.67, 1.99
Asian	11	3,796	1.53	1.13, 2.06	22.81	1.59	1.33, 1.90
European	12	5,600	2.13	1.23, 3.71	223.3	2.11	1.89, 2.36

* Data on populations of North African, Middle Eastern, Pacific Asian, and Sub-Saharan African descent were sparse and are not shown.
† CI, confidence interval.

with 9,260 alleles; Web table 2), it was 1.31 (95 percent CI: 1.02, 1.70; $Q = 83.12$, $I^2 = 86$ percent) (figure 3). Patients with type 1 diabetes were separated according to the presence or absence of GAD-65 autoantibodies in only six studies (962 alleles; Web table 3). Carriage of the **G* allele was significantly associated with the presence of these autoantibodies, without any between-study heterogeneity (random-effects OR = 1.49, 95 percent CI: 1.05, 2.13; $Q = 1.99$; $I^2 = 0$ percent) (figure 4).

Bias and heterogeneity diagnostics. There was no evidence that the magnitude of the overall odds ratio estimates changed in the same direction over time. For the allele comparison, the random-effects odds ratio was 1.44 in 1996, 1.41 in 1997, 1.39 in 1998, 1.25 in 1999, 1.30 in 2000, 1.36 in 2001, 1.48 in 2002, 1.45 in 2003, and 1.45 through October 2004.

There was evidence that larger studies (those with smaller variance) showed more conservative results for the association of the **G* allele with type 1 diabetes than smaller studies (τ rank correlation = 0.34, $p = 0.011$). This was primarily due to the three smallest studies (each with fewer than 150 subjects), which were also the ones that showed the most prominent odds ratio estimates. After exclusion of these three studies, there was no longer any significant correlation between the variance and the effect size ($\tau = 0.17$, $p = 0.22$), and the summary odds ratio by random effects was 1.33 (95 percent CI: 1.21, 1.46), with diminished between-study heterogeneity ($Q = 62.79$, $I^2 = 60$ percent).

After exclusion of both the three smallest studies and those with significant deviation from Hardy-Weinberg equilibrium, the summary random-effects odds ratio was 1.40 (95 percent CI: 1.28, 1.54), and heterogeneity decreased further ($Q = 43.62$ (21 df), $I^2 = 52$ percent). Between-study heterogeneity also diminished in the subgroups with age of onset <20 years (OR = 1.48, 95 percent CI: 1.27, 1.73; $I^2 = 57$ percent) and late onset (OR = 1.23, 95 percent CI: 0.96, 1.57; $I^2 = 79$ percent).

Other polymorphisms

The *106bp* allele of the *(AT)n* microsatellite polymorphism (four studies (2,958 alleles); random-effects OR = 0.99, 95 percent CI: 0.64, 1.55; $Q = 9.37$; $I^2 = 68$ percent) and the *T* allele of the *C(−318)T* polymorphism (three studies (2,398 alleles); random-effects OR = 0.92, 95 percent CI: 0.45, 1.89; $Q = 8.85$; $I^2 = 77$ percent) were not significantly associated with type 1 diabetes (Web table 4). Two studies (31, 49) suggested a protective effect against type 1 diabetes susceptibility for the *86bp* allele of the *(AT)n* microsatellite, but four other studies examining the *(AT)n* microsatellite (44, 50–52) did not report on this allele contrast, so the postulated association should be viewed with caution.

DISCUSSION

This meta-analysis included data from 33 studies with approximately 12,400 type 1 diabetes cases and controls. Over 10,500 subjects were from 29 studies concerning the *A49G* polymorphism. The *A49G* polymorphism is clearly associated with type 1 diabetes. We caution the reader that there was significant heterogeneity in the results of the analyzed studies. Exclusion of the smallest studies, which were also the ones that gave the most impressive estimates of association, and studies in which controls deviated from

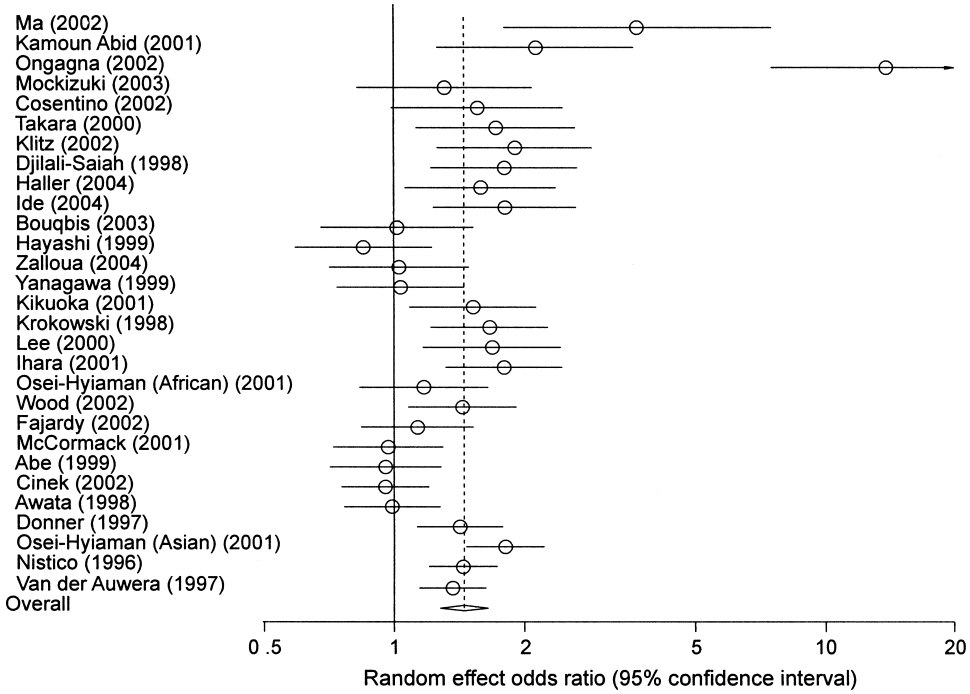

FIGURE 1. Odds ratios for the association between the **G* allele of the *A49G* polymorphism in the cytotoxic T-lymphocyte-associated antigen-4 (*CTLA-4*) gene and susceptibility to type 1 diabetes mellitus. Individual studies (circles) are listed by increasing sample size from top to bottom. The diamond shows the summary random-effects odds ratio estimate from a meta-analysis. Horizontal lines, 95% confidence interval. (For individual studies, see reference list.)

Hardy-Weinberg equilibrium diminished the heterogeneity considerably and suggested a 40 percent relative increase in the risk of type 1 diabetes conferred by this polymorphism. There were hints that the effect may be stronger in type 1 diabetics with age of onset <20 years and that the **G* allele may also be associated with the presence of GAD-65

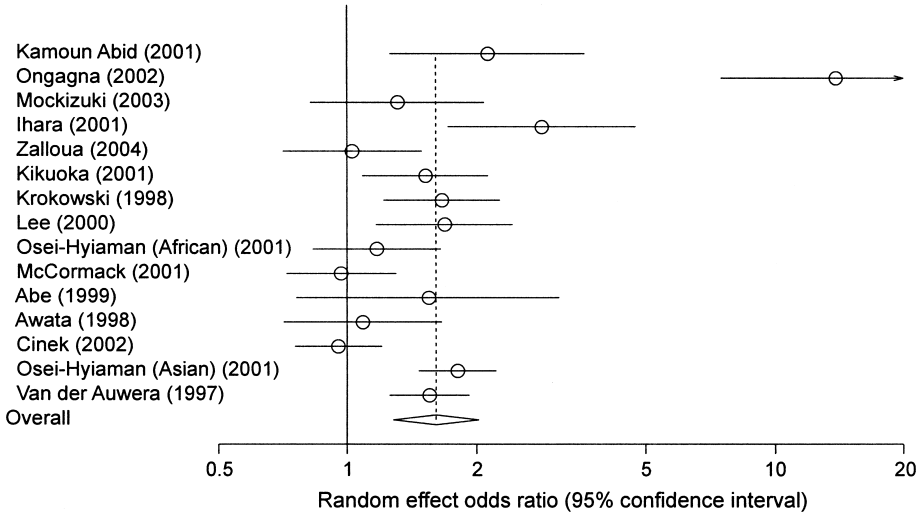

FIGURE 2. Odds ratios for the association between the **G* allele of the *A49G* polymorphism in the cytotoxic T-lymphocyte-associated antigen-4 (*CTLA-4*) gene and susceptibility to type 1 diabetes mellitus in studies or subgroups with age at onset <20 years. Individual studies (circles) are listed by increasing sample size from top to bottom. The diamond shows the summary random-effects odds ratio estimate from a meta-analysis. Horizontal lines, 95% confidence interval. (For individual studies, see reference list.)

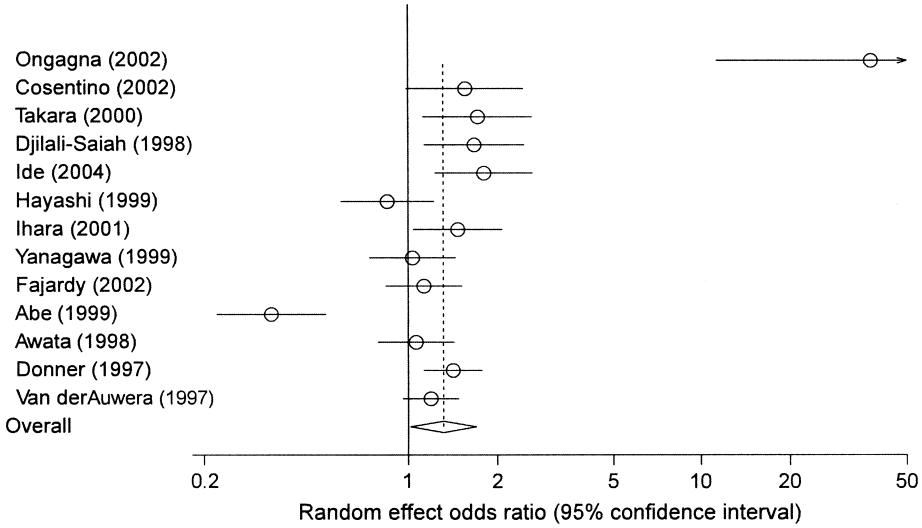

FIGURE 3. Odds ratios for the association between the **G* allele of the *A49G* polymorphism in the cytotoxic T-lymphocyte-associated antigen-4 (*CTLA-4*) gene and susceptibility to type 1 diabetes mellitus in studies or subgroups with late onset. Individual studies (circles) are listed by increasing sample size from top to bottom. The diamond shows the summary random-effects odds ratio estimate from a meta-analysis. Horizontal lines, 95% confidence interval. (For individual studies, see reference list.)

autoantibodies in type 1 diabetes patients. These latter associations should be viewed with some reservation, given the relatively wide confidence intervals and the selective reporting of information regarding these parameters in the study reports. The association of **G* allele carriage with anti-GAD-65 autoantibodies may also reflect undetected confounding with duration of disease or age of onset. However, these observations further strengthen the notion that the *A49G* polymorphism may be associated with the more typical, autoimmune variant of diabetes mellitus.

Data on other *CTLA-4* polymorphisms were sparse. We saw no effect for the *106bp* allele of the *(AT)n* polymorphism, while the *86bp* allele seemed to be associated with protection from type 1 diabetes in two studies. This would

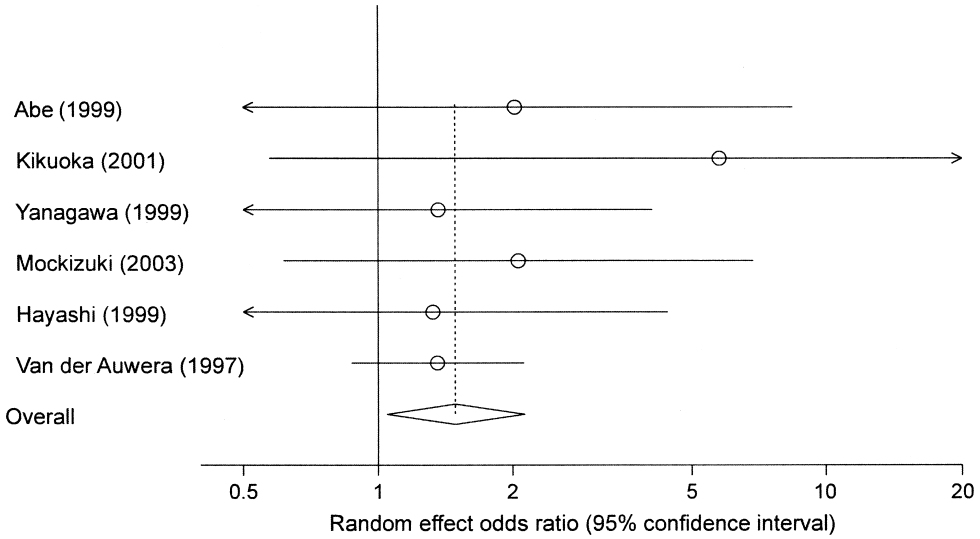

FIGURE 4. Odds ratios for the association between carriage of the **G* allele of the *A49G* polymorphism in the cytotoxic T-lymphocyte-associated antigen-4 (*CTLA-4*) gene and the presence of glutamic acid decarboxylase-65 autoantibodies among patients with type 1 diabetes mellitus. Individual studies (circles) are listed by increasing sample size from top to bottom. The diamond shows the summary random-effects odds ratio estimate from a meta-analysis. Horizontal lines, 95% confidence interval. (For individual studies, see reference list.)

be consistent with the linkage of the latter allele with the **A* allele of the *A49G* polymorphism. At least one linkage study has suggested that the polymorphism actually involved in the disease is not *A49G* but a polymorphism in linkage disequilibrium with *CTLA-4* markers that are probably closer to *(AT)n* than to *A49G* (80). Finally, because of linkage disequilibrium with the **A* allele of *A49G*, one might expect a small amount of protection to be conferred by the **T* allele of the *C(−318)T* polymorphism, and our data are compatible with this possibility.

The observed between-study heterogeneity should lead to some caution. The smallest published studies tended to produce more impressive effect sizes than the larger studies. This may be due to publication or time-lag bias, wherein smaller studies with statistically significant results tend to be published more quickly than studies of similar sample size and quality with ''negative'' results (81–83). Moreover, in five studies, genotype frequencies in controls significantly deviated from Hardy-Weinberg equilibrium. Reassuringly, after exclusion of studies that deviated from Hardy-Weinberg equilibrium as well as very small studies, the heterogeneity decreased while the risk remained the same. Still, there was considerable heterogeneity even with these exclusions. This may suggest some residual bias and/or some genuine diversity of the strength of the association, depending on the specific clinical and laboratory characteristics of type 1 diabetes. Type 1 diabetes is quite a heterogeneous syndrome, with considerable variability in age of onset, abruptness of onset, and autoantibody profile. Alternatively, heterogeneity may also point to some other ''causal'' polymorphism in variable linkage disequilibrium with the **G* allele across different populations in the same gene or in neighboring genes. Nevertheless, fine mapping analyses also suggest that peak linkage and association are observed in the *CTLA-4* region (76).

Our data are also consistent with a dose-response genetic effect: *G/G* homozygosity more than doubled the risk of type 1 diabetes. Although selecting the best genetic model is difficult, the findings of this meta-analysis are most consistent with a codominant model. Moreover, the results of the subgroup analyses for different racial descent groups were consistent. The **G* allele is considerably more common in populations of Asian descent than in populations of European descent, but the genetic effect conferred by the presence of the **G* allele seems consistent regardless of ancestry (84).

While cases and controls were not strictly matched in most of the studies reviewed, this is unlikely to have introduced considerable bias in the meta-analysis. Minor potential geographic mismatching would probably not be very important. There were also differences in the extent of testing to exclude controls with type 1 diabetes, and in many studies screening and ascertainment of subjects in the control group were not described in detail. However, missed type 1 diabetes is unlikely, except in the early subclinical stages, and the proportion of healthy children who might subsequently develop type 1 diabetes during their lives is also likely to be negligible. For cases, diagnosis of type 1 diabetes is also quite straightforward—although several studies did not provide enough detail on the definition of type 1 diabetes, and thus some misclassification of type 2 diabetes as type 1 diabetes (especially in Asian youths) cannot be fully excluded. This being acknowledged, overall misclassification of cases and controls is probably not a major issue in this meta-analysis.

Because of the various and serious lifelong complications of type 1 diabetes, it is crucial to identify etiologic factors for the pathogenesis of this disease. The major histocompatibility complex region explains approximately half of the genetic susceptibility to type 1 diabetes (65, 66), suggesting that additional determinants exist, and such determinants are suggested repeatedly by different genome scans (67–73, 85). However, while the list of identified candidate susceptibility loci is continuously expanding, most of the reported associations to date remain nonreplicable or at least controversial after subsequent investigation. Even when genetic associations are replicated (86), they usually have a minor public health impact that would not lead to routine screening recommendations (87). Nevertheless, such knowledge could improve our understanding of complex and multivariate diseases such as type 1 diabetes. Allowing for these caveats, the current meta-analysis demonstrates that *CTLA-4* is a genetic determinant of type 1 diabetes.

LABORATORY TESTS

The methods used for *CTLA-4 A49G* genotyping in the studies analyzed here were based on the polymerase chain reaction technique, but several different variants were used, and primers differed across studies. The most common primers were forward 5′-GCTCTACTTCCTGAAGACCT-3′ and reverse 5′-AGTCTCACTCACCTTTGCAG-3′ (used in eight studies); forward 5′-GCTCTACTTCCTGAAGACCT-3′ and reverse 5′-AACCCAGGTAGGAGAAACAC-3′ (used in six studies); and forward 5′-CCACGGCTTCCTTTCTC-GTA-3′ and reverse 5′-AGTCTCACTCACCTTTGCAG-3′ (used in four studies). Other primers were used in six studies, and in four studies the researchers did not specify the primers used. Although the genotyping error rate is likely to be small for a biallelic polymorphism, none of the studies included in the meta-analysis reported any data on validation of the genotyping or estimation of the measurement error rate. Interlaboratory standardization and consistent reporting of results may be an even greater issue for the microsatellite polymorphism. Future studies should also ensure and clearly report that the personnel who performed the genotyping were blinded as to the clinical status of participants.

POPULATION TESTING

To date, no population testing for *CTLA-4* polymorphisms is in use. The results of this meta-analysis do not suggest that such testing is indicated on a population-wide basis, given the relatively modest increase in risk conferred by the **G* allele. Nevertheless, in future studies, investigators should consider including screening for the *CTLA-4 *G* allele in randomized clinical trials that enroll children at moderate or high perceived risk of developing type 1 diabetes. Family history remains the strongest risk factor for

type 1 diabetes susceptibility, but trials have also used immunologic testing for the presence of autoantibodies, metabolic screening, and human leukocyte antigen genotyping in various combinations (88–91). *CTLA-4* genotyping may also provide some useful additional information for identifying high-risk children. On the basis of the frequency of the **G* allele in the analyzed studies and the estimated odds ratio, one can calculate that the attributable fraction of type 1 diabetes due to this polymorphism is 18 percent in populations of Asian descent and 16 percent in populations of European descent. We acknowledge that prior randomized trials on primary prevention using other parameters for risk stratification have either had largely negative results (88, 89) or are ongoing (91), but the concept of early intervention should be probed further with different preventive interventions and better risk stratification.

ACKNOWLEDGMENTS

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